

Compound isolated from the cock's comb and its effect on palmar arsenical keratosis

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Abstract

This study was conducted to confirm the effectiveness of the ointment containing cock's comb extract and to identify the compound that was effective in the treatment of palmar arsenical keratosis. On the basis of inclusion and exclusion criteria, 24 patients with moderate to severe palmar arsenical keratosis were enrolled from an arsenic endemic area. The ointment prepared from the cock's comb extract was supplied to each patient at an interval of 2 weeks. Each patient was advised to apply the ointment on the nodule by rubbing gently up to 5 minutes and twice daily for 12 weeks. Improvement was monitored by measuring the nodular size before starting and after completion of the treatment. The mean (\pm SD) size of the nodules of the palm before treatment was 16.6 ± 5.0 mm², which was reduced to 4.0 ± 3.8 mm² after completion of the treatment. This reduction was statistically significant. To identify the effective compound nuclear magnetic resonance spectroscopy, infrared spectrophotometry, elemental analysis and liquid chromatography-mass spectroscopy of the extract were done yet conclusion could not be achieved.

Introduction

Arsenical keratosis is not only a health problem but also a social hazard in Bangladesh.¹ The poor woman is the worst affected patient. The husband of an arsenicosis patient often refuses to keep the marital relationship. The parents of an affected marriageable girl get difficulty to find out a groom.² Despite the magnitude of the problem, there is no effective therapy for the treatment of keratosis.³ Several topical and oral formulations were applied for the treatment of keratosis. However, the beneficial effects of these agents are not established.

A study conducted in this department to see the effectiveness of cock's comb extract in the treatment of palmar arsenical keratosis and was found effective, without any adverse effect.⁴ Cock's comb is a big flap of skin. From ancient times, combs were used as food. People in Western Europe and China considered combs as a royal food.⁵ Cock's comb consists of hyaluronic acid, chondroitin sulfate, dermatan sulfate and heparan sulfate. The normal desquamation process of the skin is disrupted in keratosis. Desquamation of the skin is maintained by continuous hydration of the stratum corneum.⁶ As an occlusive agent hyaluronic acid prevents transepidermal fluid loss.⁷ Chondroitin sulfate also contributes to the hydration of the tissue.⁸

So, the purpose of this study was to confirm the

beneficial effect of the cock's comb extract and to identify the compound that was effective in the treatment of palmar arsenical keratosis. Extraction of cock's comb on a large scale is difficult, time-consuming and expensive. By identifying the compound, the chemical synthesis will be possible. This may cause the availability of medicine at low cost for the arsenic affected population.

Materials and Methods

The study was conducted in four places: a) Department of Pharmacology, where extraction and purification of cock's comb, its cytotoxic effects on brine shrimp, ointment preparation, liquid chromatography-mass spectroscopy of the extract and estimation of arsenic in drinking water and nails of the patients were done, b) Patients were enrolled from the Kamalla Union of Muradnagar Upazilla at Cumilla District, c) NMR and IR of the extract were done at the Wazed Miah Science Research Center, Jahangir Nagar University, Savar, Dhaka, d) Elemental analysis of the extract was done at the Bangladesh Council for Scientific and Industrial Research, Dhaka.

Enrollment of patients

This study was carried out on 24 patients (12 males and 12 females) of palmar arsenical keratosis from September 2017 to January 2019.

They voluntarily agreed to participate and they were enrolled according to the inclusion and exclusion criteria. Patients aged >18 or <65 years of both sex with palmar arsenical keratosis, and consumption of arsenic water (>50 µg/L) for more than 6 months were enrolled. An expectant or nursing mother, patient having drug or food allergy especially to avian protein, the patient who received any treatment of arsenicosis within the last 3 months were not enrolled.

Patient's data collection procedure

The detailed information was taken by interviewing the patients and clinical examinations were done mainly based on arsenical keratosis of the palm of the patients and documented in the case report form.

Measurement of the size of lesion

The size of the lesion was measured before starting the treatment and 12 weeks after the completion of treatment. Photographs of the lesions were also collected both before and after completion of treatment.

Collection of samples

To confirm the diagnosis, the water samples as well as the nails were collected before starting the treatment for the estimation of total arsenic level.

Estimation of arsenic

Estimation of arsenic was done by the modified silver diethyldithiocarbamate method (SDDC).⁹

Collection of cock's comb

The cock's combs (12 kg) were collected from the poultry shop of a local market and stored in (-20°C) until extraction.

Method of cock's comb extraction¹⁰

The combs were squeezed by aqueous separation process. About 500 g cock's combs were sliced at a time into small pieces. The pieces of combs were soaked in 1L of acetone for 24 hours and kept in a refrigerator. After 24 hours, the acetone was squeezed from the materials of the comb and again soaked in 1L of acetone for the next 24 hours. This step was repeated four times. After the last extraction, the remaining acetone was dried up in a stream of air. The weight of the dried and defatted combs was about 110 g. These dried combs were submerged in 5% of 500 mL solution of sodium acetate for another 24 hours. After 24 hours, the viscous fluid was drawn out by squeezing the sliced combs and then filtered by using normal filter paper. This process was repeated four times. The final comb residue was then disposed of. Ethyl alcohol was added to the aqueous extract. The precipitate formed was pooled, centrifuged, re-dissolved in 5% sodium acetate solution and re-centrifuged. The protein was

removed from the supernatant solution by shaking it with chloroform. Then chloroform-amyl alcohol (1:4 parts) mixture was added several times until a gel no longer formed. The solution was acidified to pH 4.0 and precipitated with ethyl alcohol. After filtration, the precipitate was collected from the filter paper and was weighted about 5.0 g. The solid extract was brown and dissolved in *n*-butanol. According to this procedure, extract was prepared several times. From 12 kg of cock's comb, 140 g extract was obtained.

Brine shrimp lethality test¹¹

Artificial seawater was prepared into a square-shaped jar by dissolving 27 g sodium chloride in 3 L of tap water. About 3 g of brine shrimp eggs (collected from local market) were poured into the jar for hatching with continuous aeration by airflow machine and artificial light using 60-watt bulb. After 48 hours, the brine shrimp larvae (nauplii) were collected with a Pasteur pipette into five sterile test tubes labeled as I-V.

One milligram of the extract was weighted by an analytical balance. The stock solution was prepared by dissolving the extract in 1 mL of water. To prepare the different concentrations (100, 10, 1.0, 0.1 µg/mL), serial dilution of the stock solution was done. About 1 mL of the prepared solution was taken into each test tube containing actively moving 10 live nauplii in 1 mL of artificial seawater. The test tubes containing live nauplii were incubated at room temperature. The active movement of nauplii was observed after 2 and 24 hours.

$$\% \text{Death} = \frac{\# \text{Dead nauplii}}{\# \text{Dead nauplii} + \# \text{Live nauplii}} \times 100$$

Preparation of ointment¹²

All the ingredients required to prepare ointment were measured by an analytical balance. A medium -size bowl containing 100 mL of water was heated up to boiling by a Bunsen burner. A large beaker containing white petroleum (86 g), stearyl alcohol (3.9 g) and bee wax (0.08 g) was placed into the bowl. After the complete melting of all the ingredients, the burner was put off. The beaker was cool down at room temperature. The cock's comb extract (0.1 g) was added and continuously beat until it became thick. Finally, it was poured into the high-density polyethylene container (25 g each) and stored in a cool and dry place until supplying the patient.

Distribution of ointment and periodic monitoring

Every 2 weeks interval, the ointment was distributed to each patient. The instruction was given how to apply the ointment twice daily over the keratotic lesion of the palm by gently rubbing up to

Table I

Percentage of dead nauplii at different concentrations of the extract		
Concentration of extract (µg/mL)	%Dead nauplii	%Net dead nauplii*
0	54 ± 05	
1	90 ± 14	36
10	86 ± 16	32
100	84 ± 20	30
1000	92 ± 13	38

Data are presented as mean ± SD; *after subtraction of control value

5 min. A printed compliance sheet was given to give a tick mark after applying the ointment. The adherence of the patient and any change in lesion size were monitored by the researcher.

Steps of identification

The compound was identified by thin layer chromatography, column chromatography, nuclear magnetic resonance spectroscopy, fourier transform infrared spectrophotometry, elemental analysis and liquid chromatography-mass spectroscopy. The light brown peak observed in the thin layer chromatography in a solvent system of chloroform : *n*-hexane (3:3). The light brown compound was obtained from column chromatography. This separated compound was dried up with the help of argon gas and collected in Eppendorf tube for NMR, FTIR, elemental analysis and LC-MS. The NMR and FTIR spectra of the cock's comb extract were compared with the reference standard.

Statistical analysis

Paired 't' test was done to compare the lesion size before and after 12 weeks of treatment and data were presented as mean ± SD.

Results

The extract was brown in color, solid in consistency, pungent in smell and soluble in *n*-butanol, but insoluble in methanol, ethanol, chloroform and water. The net dead nauplii were 36% at 1 µg/mL concentration (Table I).

The mean concentration of arsenic in the fingernail of the patients was 7.5 µg/g. The mean concentration of arsenic in the tube well water of the patients was 146 µg/L.

Clinical improvement

The mean size of the keratotic nodules before treatment was 16.6 mm² which reduced to 4 mm² after 12 weeks of the study (Table II). The p value

Table II

Size of keratosis before and after applying ointment		
Patient	Before (mm ²)	After (mm ²)
1	25.8	5.9
2	20.2	2.0
3	22.7	1.3
4	10.1	3.6
5	16.6	16.6
6	10.5	0.0
7	19.7	2.7
8	17.6	11.1
9	12.0	4.0
10	12.5	6.0
11	15.0	0.0
12	16.2	1.0
13	16.2	0.0
14	19.9	5.5
15	19.5	0.0
16	11.2	1.0
17	10.1	3.8
18	17.7	4.0
19	15.6	4.2
20	10.5	1.0
21	14.5	4.0
22	25.6	6.0
23	25.6	6.0
24	14.5	5.1
Mean ± SD	16.6 ± 5.0	4.0 ± 3.8

was <0.00001. The net reduction was about 75%.

Characteristics of the extract

The compound yield was 1.2%. The retention factor was 0.26. The mass was 412. The elemental analysis showed 51% carbon and 8% hydrogen. The spectra of FTIR and NMR were compared with the reference standard.

Comparison of wavelength of FTIR spectra with standard reference

Only two waves number were found similar to the standard references those are 1618 and 2924 both for functional group amine (Table III).

Comparison of chemical shifts of ¹³C-NMR with standard reference

The chemical shifts were found similar only at the 179, 103 and 23 ppm (Table IV).

Comparison of chemical shifts of ¹H -NMR with standard reference

The chemical shift was found similar at 2 ppm (Table IV).

Table III**Comparison of infrared spectra of the extract with standard references**

Wavelength (cm ⁻¹)		
Present study	Hafsa et al., 2016	Reddy and Karunakaran, 2013
1099	1082	1041
1165	1150	
1379	1324	
1419	1409	1413
1618	1617	1619
	1650	
2854		2896
2924	2925	
3003		
3415	3390	3424
3469		
3547		

However, 100% improvement was not observed in this study. Perhaps by increasing the duration of treatment or frequency of administration, 100% improvement might have been achieved.

There were several studies conducted to assess the effectiveness of different treatment options applied in patients with palmar arsenical keratosis. In 2007, a study showed there was a significant decrease in blood arsenic level after the supplementation of folic acid.¹³ Arsenic is methylated to monomethyl-arsenic and dimethylarsenic acids on folate-dependent metabolism, as a result, increases urinary arsenic excretion. But folic acid may cause nausea, vomiting, skin rashes and depression.

A trial was conducted to show the effectiveness of oral administration of a low dose of acitretin in combination with salicylic acid.¹⁴ In that trial, severe mucocutaneous adverse effect was reported with acitretin.

Another study showed the effectiveness of the oral administration of garlic oil for the treatment of mild to moderate degree of palmar arsenical keratosis.¹⁵ In which, garlic oil was administered in the form soft capsule (10 mg) daily for 12 weeks. The study found that there was a reduction of the total accumulated arsenic from the body with the improvement of the nodular size. But, treatment with garlic caused several adverse effects such as gastric irritation, vertigo, diarrhea, and constipation. Besides, the smell was a common discomfort in the treatment with garlic.

In 2014, a study was conducted to show the effect of

Discussion

Topical application of the cock's comb extract on patients with moderate to severe palmar arsenical keratosis was found effective by measuring the nodular size before and after the completion of the treatment. This is the second study to evaluate the effects of the cock's comb extract on arsenical keratosis. In the previous study,⁴ there was 84% reduction of the nodular size. In the present study, 75% reduction of the size of the nodule was observed. The study was statistically significant.

Table IV**Comparison of chemical shifts (δ in ppm) of ¹³C NMR spectra of the extract with standard references**

¹³ C NMR spectra						
Present study		Rosa et al., 2012		Bociek et al., 1980		
N-acetyl glucosamine	Glucuronic acid	N - acetyl glucosamine	Glucuronic acid	N - acetyl glucosamine	Glucuronic acid	
	179		176		177	
103	105	103	106	103	104	
		71		71		
65		63		62		
50		57		56		
23		25		23		
¹ H NMR spectra						
Present study		Welti et al., 1978		Wende et al., 2016		
4.46	4.29	4.5	4.4	5.1	5.1	
3.65	3.43	3.8	3.3	4.0	3.8	
			3.5	4.0	4.0	
			3.7	3.5	5.9	
		3.4		3.9		
2.0		2.0		2.1		

Nigella sativa on palmar arsenical keratosis.¹⁶ The disadvantage of that study was maximum patients suffered from gastric irritation during the study period.

Another study evaluated the effectiveness of the topical administration of salicylic acid for the treatment of palmar arsenical keratosis.¹⁷ An ointment containing 20% and 30% of salicylic acid had been found more effective. There were burning sensation and pruritus due to long-term administration of a high concentration of salicylic acid.

A trial was conducted to evaluate the effectiveness of the topical administration of propylene glycol in the treatment of palmar arsenical keratosis.¹⁸ The disadvantage of that study was itching of the patients.

A study was conducted to evaluate the effect of the extract of leaves of *Azadirachta indica* on the palmar arsenical keratosis.¹⁹ The common problems of that study were foul-smelling and burning sensation.

In the present study, the effectiveness of the topical administration of cock's comb extract was further evaluated. Cock's comb composed of hyaluronic acid, chondroitin sulfate, dermatan sulfate and heparan sulfate.²⁰⁻²²

The topical administration of ointment containing cock's comb extract maintains the hydrophilic state in the stratum corneum. By retaining a large amount of water it keeps the skin much more hydrated and repeated hydration regulates the desquamation process. The intracellular lipids also regulate the normal desquamation process, which is also maintained by the hydration of the stratum corneum.²³ In case of keratosis, excess deposition of the keratin occurs and the natural desquamation process is hampered. It can be considered that the administration of ointment containing cock's comb extract reduces the size of the keratosis by desquamation process and can be used effectively in the treatment of palmar arsenical keratosis.

Chondroitin sulfate is another component of the cock's comb. It also contributes to the hydration of the tissue; inhibits the release of free reactive oxygen species, nitric oxide.⁸

Dermatan sulfate plays a critical role in the development of the central nervous system, wound repair, infection control, morphogenesis and cell division.²⁴

Heparan sulfate can attach extracellular superoxide dismutase to the endothelial cell surface and thus play a role as an antioxidant. It also activates antithrombin III which inhibits the coagulation process at several steps.²⁵

This study showed that the ointment containing cock's comb extract was found effective in the treatment of moderate to severe arsenical keratosis, without any systemic adverse effects. To identify

the effective compound, nuclear magnetic resonance spectroscopy, fourier-transform infrared spectrophotometry, elemental analysis and liquid chromatography-mass spectrometry of the extract were done.

Several studies were conducted to identify hyaluronic acid extracted from different sources. In 2003, a study was conducted to purify hyaluronic acid from the mollusc bivalve *Mytilus galloprovincialis*, using ¹³C nuclear magnetic resonance spectroscopy.²⁶ In that study, the spectra for glucuronic acid units were observed at 106.0 ppm for C-1, 75.3 ppm for C-2, 76.3 ppm for C-3, 82.7 ppm for C-4, 79.0 ppm for C-5, and 177.0 ppm for C=O. The spectra observed for n-acetylglucosamine units were at 103.5 ppm, 57.1 ppm, 85.3 ppm, 71.2 ppm, 78.5 ppm, 68.3 ppm for C-1, C-2, C-3, C-4, C-5, and C-6 respectively.

Another study was conducted with the human umbilical cord,²⁷ and identified carboxyl and acetamide groups at 173.4 and 144.5 ppm, respectively, two anomeric carbons at 100.1 and 102.7 ppm and an acetamide carbon at 22.1 ppm. The signals observed at 53.9 and 60.4 ppm for the C2 and C6 carbons of the glucosamine residue.

In 2012, another study was conducted to identify hyaluronic acid extracted from the cock's comb.²⁸ The ¹³C nuclear magnetic resonance spectra of that study found that for glucuronic acid units the chemical shifts were observed at 177.0 ppm for COO-, at 106.0 ppm for C-1, at 83.0 ppm for C-4, at 79.4 ppm for C-5, at 76.7 ppm for C-3 and C-2 at 75.6 ppm. For the n-acetylglucosamine units, a signal was observed at 178.0 ppm for the C-2 carbons, and signals identified at 103.4, 85.5, 78.4, 71.6, 63.5 and 57.3 ppm corresponding to the C-1, C-3, C-5, C-4, C-6 and C-2 carbons, respectively. At 25.5 ppm, a characteristic shift for the methylic carbon was observed. In the ¹³C nuclear magnetic resonance spectra of this study signals observed for glucuronic acid units were at 105.8 ppm for C-1, and signal observed at 179.3 for C=O. The chemical shifts observed for n-acetylglucosamine units were at 103.2 ppm, 50.8 ppm, and 65.5 ppm, corresponding to C-1, C-2, C-3. A characteristic methylic carbon was observed at 23.0 ppm.

¹H-magnetic resonance is another useful tool for the identification of hyaluronic acid. In 1979, a study was conducted to compare the conformational changes of spectra of hyaluronic acid chondroitin sulfate that occur in alkaline solution.²⁹ In that study, spectra observed in neutral solution were as follows: at 4.4 ppm, 3.3 ppm, 3.5 ppm and 3.7 ppm for H-1, H-2, H-3, and H-4 respectively for the glucuronic acid units. Spectra observed for n-acetylglucosamine units were as follows: at 4.5 ppm, 3.8 ppm, 3.7 ppm, 3.5 ppm, 3.4 ppm and 4.0 ppm for H-1, H-2, H-3, H-4, H-5, H-6 and H-6' respectively. A spectrum observed at 2.0 ppm for

acetamidomethyl proton.

Another study was conducted to determine hyaluronic acid using NMR and mass spectrometry-based methods.³⁰ In that study, ¹H-magnetic resonance spectra for a) glucuronic acid units observed at 5.1 ppm, 3.8 ppm, 4.0 ppm, 6.0 ppm for H-1, H-2, H-3, and H-4 respectively and spectra for b) n-acetylglucosamine units observed at 5.1 ppm, 4.0 ppm, 4.0 ppm, 3.5 ppm, 4.0 ppm, 3.8 ppm, and 3.8 ppm for H-1, H-2, H-3, H-4, H-5, H-6 and H-6' respectively. A spectrum observed at 2.1 ppm for acetamidomethyl proton.

On the other hand, ¹H NMR spectra of this study were as follows: - For glucuronic acid units- at 4.29 ppm and 3.43 ppm for H-1 and H-2 respectively. For n-acetylglucosamine units- at 4.46 ppm and 3.65 ppm for H-1, H-2 respectively and spectrum observed at 2.0 ppm for acetamidomethyl proton.

Characterization of hyaluronic acid by the fourier transform infrared spectroscopy is another useful method. A study was conducted to evaluate the effect of ultrasonic degradation of hyaluronic acid extracted from rooster comb on antioxidant and antiglycation activities.³¹ In that study, the main spectra of the fourier transform infrared spectroscopy indicate an O-H and N-H stretching vibrations at 3390 cm⁻¹, C-H stretching vibrations at 2925 cm⁻¹. The spectra at 1617 and 1409 cm⁻¹ were due to N-H and C-O stretching modes of the planar carboxyl groups. The absorption spectra about 1650, 1551 and 1324 cm⁻¹ correspond to the amide I, II and III, respectively, C-O-C group at 1150 cm⁻¹ (O-bridge) and the C-O-H group at 1041 cm⁻¹.

Another study was conducted where purification and characterization of hyaluronic acid were produced by *Streptococcus zooepidemicus* strain 3523-7.³² In that study, the significant spectra (cm⁻¹) were at 611.3, at 1043.3 that could be due to the C-O-C stretching, at 1411.6 that corresponds to the presence of C-O group with C=O combination, at 1616.0 that indicates the presence of amid II group, at 2892.7 due to the C-H stretching and at 3407.6 that confirms the presence of OH stretching.

In the present study, the spectra of fourier transform infrared spectroscopy (cm⁻¹) of cock's comb extract are at 3547.0 due to O-H stretching, at 3469.0 due to N-H stretching, at 3003.1 due to O-H stretching for carboxylic acid, at 2854.6 due to O-H stretching for alcohol, peak at 1618.0 due to N-H stretching and at 1165.0 due to C-O stretching for ester.

The nuclear magnetic resonance (¹³C and ¹H) and the fourier transform infrared spectrophotometry data of this study were not consistent with that of previous studies, conducted to identify hyaluronic acid extracted from a different source and could not support the presence of hyaluronic acid in this extract.

Elemental analysis of the extract was done but the conclusion was not achieved. The report showed that there was 51.4% carbon and 8.13% hydrogen. Nitrogen was not detectable in the extract and there was a lack of facility to obtain the percentage of oxygen and sulfur. From the molecular structure of hyaluronic acid, chondroitin sulfate, dermatan sulfate and heparan sulfate, it was revealed that all of them contain nitrogen. Anal. Calcd for C₂₈H₄₄N₂O₂₃ (hyaluronic acid): C, 43.41; H, 5.42; N, 3.61; O, 47.54; Anal. Calcd for C₁₄H₂₁NO₁₅S (dermatan sulfate): C, 34.35; H, 4.29; N, 2.94; O, 49.07; S, 6.54; Anal. Calcd for C₁₃H₂₁NO₁₅S (chondroitin sulfate): C, 33.69; H, 4.53; N, 3.02; O, 51.83; S, 6.91; Anal. Calcd for C₁₄H₂₃NO₂₁S₃ (heparan sulfate): C, 26.37; H, 3.61; N, 2.19; O, 52.74; S, 15.07.

In the liquid chromatography-mass spectroscopy method, the mass of the extract was found 412.8 Dalton. The molecular mass of hyaluronic acid, heparan sulfate, dermatan sulfate and chondroitin sulfate are about 776 Dalton, 637 Dalton, 475.37 Dalton and 463.36 Dalton respectively.

Conclusion

The present study confirms the effectiveness of the cock's comb extract in the treatment of palmar arsenical keratosis. By analyzing the NMR, FTIR, elemental analysis and LC-MS, it can be said that the effective compound was something else other than hyaluronic acid, heparan sulfate, dermatan sulfate and chondroitin sulfate.

Ethical Issue

The protocol was approved by the Institutional Review Board of Bangabandhu Sheikh Mujib Medical University (Reg. No. BSMMU/2018/2966). This study was registered on the clinical trial.gov. the registration No. was NCT03635853. The participants were informed about the risks, benefits and possible outcomes of this work and the information were given in an easy and local language (Bengali). Informed written consent was taken from each patient.

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